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Protein Digestion: An Overview of the Available Techniques and Recent Developments

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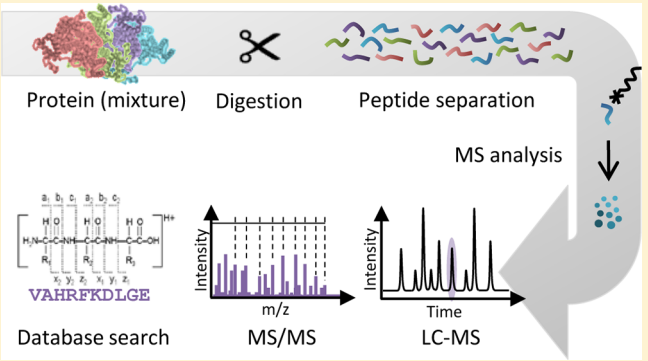
ABSTRACT: Several proteomics approaches are available that are defined by the level (protein or peptide) at which analysis takes place. The most widely applied method still is bottom-up proteomics where the protein is digested into peptides that can be efficiently analyzed with a wide range of LC−MS or MALDI-TOF-MS instruments. Sample preparation for bottom-up proteomics experiments requires several treatment steps in order to get from the protein to the peptide level and can be very laborious. The most crucial step in such approaches is the protein digestion, which is often the bottleneck in terms of time consumption. Therefore, a significant gain in throughput may be obtained by speeding up the digestion process. Current techniques allow for reduction of the digestion time from overnight (~15 h) to minutes or even seconds. This advancement also makes integration into online systems feasible, thereby reducing the number of tedious sample handling steps and the risk of sample loss. In this review, an overview is given of the currently available digestion strategies and recent developments in the acceleration of the digestion process. Additionally, tailored approaches for classes of proteins that pose specific challenges are discussed.



KEYWORDS: *protein digestion, accelerated digestion, bottom-up proteomics, enzymatic digestion, chemical digestion, IMER, membrane proteins, PTM*

1. INTRODUCTION

Protein digestion, either enzymatically or nonenzymatically, is an important and (almost) indispensable tool in protein identification, characterization, and quantification by proteomics strategies.[1] Proteomics plays a vital role in major research areas, including disease biomarker discovery and systems biology, and, as such, significantly contributes to the understanding of biological processes that are essential for life.[2]

Selection of the proteomics approach should be based on the type of question to be answered. Global proteomics, such as finding a biomarker in a highly complex sample, concerns the identification of an often low abundant, unknown protein that is present in a complex sample with a wide dynamic range of proteins. This requires a very different approach than the targeted analysis of a protein, for example, characterization of post-translational modification (PTM) states, where a detailed investigation and complete sequence mapping of a known protein is required for localization of the possibly low-abundant PTMs. Additionally, (classes of) proteins with special characteristics may pose specific requirements in their analysis.

Proteomics approaches can be discriminated by the level at which analysis takes place (see Figure 1). Advances in mass spectrometry (MS) instrumentation now allow for the direct analysis of proteins. In such a so-called top-down experiment, purified proteins are detected intact and following fragmentation using collisional-activated dissociation (CAD), electron-capture dissociation (ECD), or electron-transfer dissociation (ETD), providing information on intact protein mass and amino acid sequence.[3,4] Top-down analysis of intact proteins reduces sample preparation to a minimum and preserves information that is sometimes lost in other proteomics strategies, such as the connectivity of multiple PTMs, but is relatively insensitive.[5] Due to the large size of the analytes, the requirements for MS instruments in terms of resolution and mass accuracy are only provided by high-end mass spectrometers such as the Fourier transform ion-cyclotron resonance MS (FTICR-MS) and Orbitrap MS.[6] However,

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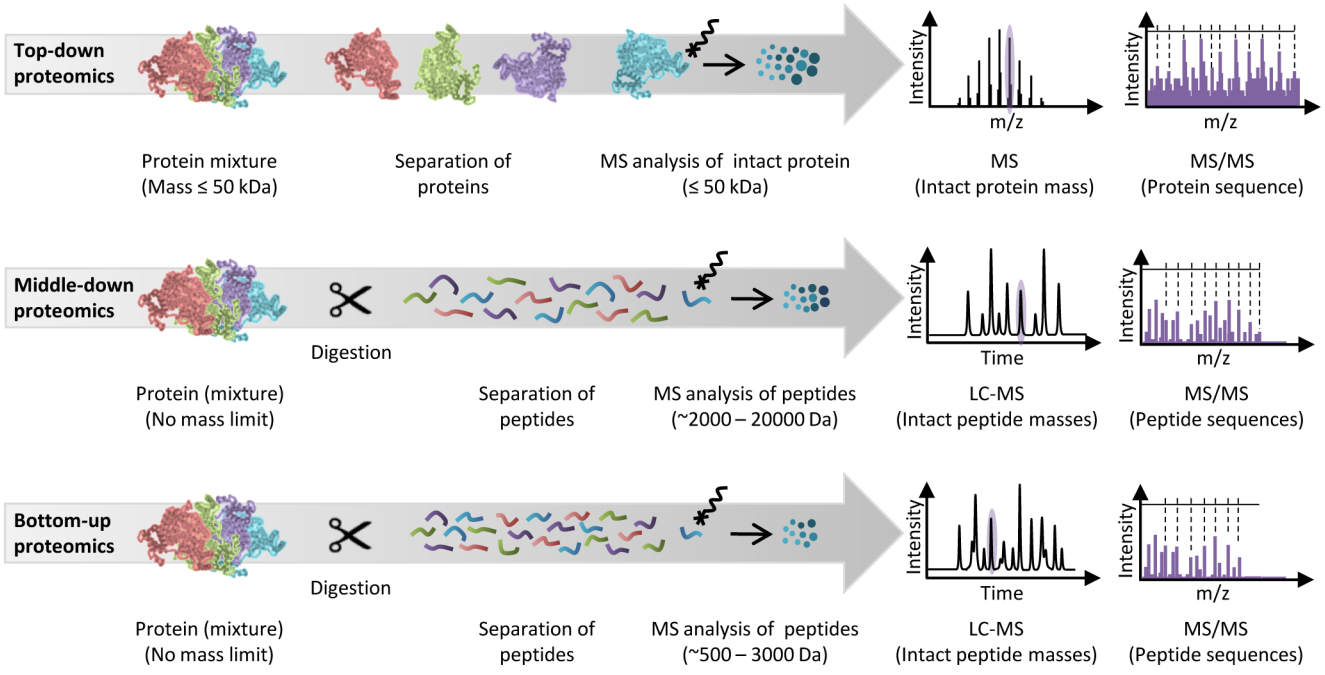

Figure 1. Overview of the proteomics approaches.

intact protein analyses have also been reported using more accessible instruments, such as the tandem quadruple and quadrupole-time-of-flight (QTOF) MS.[7] In practice, the mass range of proteins that can be analyzed using top-down proteomics is limited to ~50 kDa, thus approximately 500 amino acids.[6] Otherwise, only the C- and N-termini are sequenced.[8,9] Despite the clear advantages of top-down proteomics, further development of MS instrumentation is necessary before it will become a mainstream technique.

The vast majority of proteomics experiments rely on digestion of the protein into peptides prior to MS analysis, which is the main focus of this review. The analysis of peptides has several advantages over proteins, including more efficient separation by liquid chromatography (LC), a lower molecular mass and fewer charge states, leading to improvements in sensitivity.[10] Depending on the size of the produced peptides, the approach is referred to as either bottom-up proteomics or middle-down proteomics. In a bottom-up strategy, the protein is digested to peptides within the range of ~500−3000 Da. These peptides are subsequently analyzed with liquid chromatography−electrospray ionization MS (LC−ESI-MS) or matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS). Protein identification is performed based on peptide mass fingerprinting or peptide sequence analysis.[3] Sequencing of the peptides can effectively be achieved by collision-induced dissociation (CID) in more widely available ESI-ion-trap and ESI-QTOF mass spectrometers or using MALDI-TOF/TOF instruments.[11]

Digestion of a complex protein sample, for example, a whole proteome, with a bottom-up approach produces a vast amount of peptides, more than even the most efficient instrument can analyze. The peptide sample complexity can be reduced without compromising the information content by producing fewer, but larger peptides. This, so-called middle-down proteomics approach combines the best of top-down and bottom-up by taking advantage of the improvements in MS instrumentation and the availability of electron-based fragmentation methods,[12] while retaining the level of sensitivity associated with the analysis of peptides.[5] The midrange peptides (~3000−20000 Da) show improved separation by LC,[13] and after ESI carry a higher number of charges, which enhances fragmentation by CID,[13] ETD[14] or ECD[15] in Orbitrap MS, quadrupole-linear ion trap (QTrap) MS and quadrupole- FTICR-MS instruments. In comparison with smaller peptides, more confident peptide identifications are obtained, leading to improved protein sequence coverage and identification of PTMs.[16]

The predictable nature of peptide fragmentation allows matching of experimental MS/MS spectra with predicted spectra from *in silico* digestion of known protein sequences to establish protein identity.[17] In that respect, advanced bioinformatics tools play a pivotal role in any proteomics strategies. However, these tools rely on the assumption that the digestion process (including reduction and alkylation of disulfide bridges) is optimal. If this is not the case, for example, when peptides are connected via an intact disulfide bridge, unpredicted peptides and/or complex fragmentation spectra are generated that will not be identified in an automated database search. Digestion of proteins may result in a loss of information, such as, the presence and connectivity of PTMs[7] or the ability to distinguish closely related proteins, because of failure in detection of certain parts of the protein sequence due to inadequate size or unfavorable ionization properties of the generated peptides. Finally, the quality of the obtained protein identifications and modifications can be monitored via false discovery rates, protein and peptide score thresholds, but this requires a critical review of the obtained results.

Protein digestion is an essential step in both bottom-up and middle-down proteomics strategies and has a large influence on the quality of protein identification.[18] Over the years, protein digestion has been improved through the development of novel techniques in order to increase throughput and reproducibility. This paper provides an overview of the available protein

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digestion techniques and reviews recent developments in protein digestion for proteomics.

2. PROTEIN DIGESTION

The classical approaches for protein digestion are enzymatic digestion, involving proteolytic enzymes, and nonenzymatic digestion, utilizing chemicals, and are mostly performed in-solution or in-gel. A wide range of proteolytic enzymes with varying cleavage specificities and efficiencies is available for enzymatic digestion to which new enzymes are added regularly. Chemical digestion mainly utilizes acids and small chemical reagents, but recently also instrumental techniques have been introduced as a method for nonenzymatic digestion. An overview of the available approaches is given in this chapter.

2.1. Enzymatic Digestion

The most widely applied method for protein digestion involves the use of enzymes. Many proteases are available for this purpose, each having their own characteristics in terms of specificity, efficiency and optimum digestion conditions. Trypsin is most widely applied in bottom-up proteomics and can be considered as the gold standard in proteomics. Over the years, this enzyme has been modified to a highly efficient and autolysis-resistant protease.[19] It is available in large quantities at low cost and has a very high degree of specificity, cleaving the peptide bonds C-terminal to the basic residues Lys and Arg, except when followed by Pro.[20] Lys and Arg are relatively abundant amino acids in the human proteome (see Figure 2)

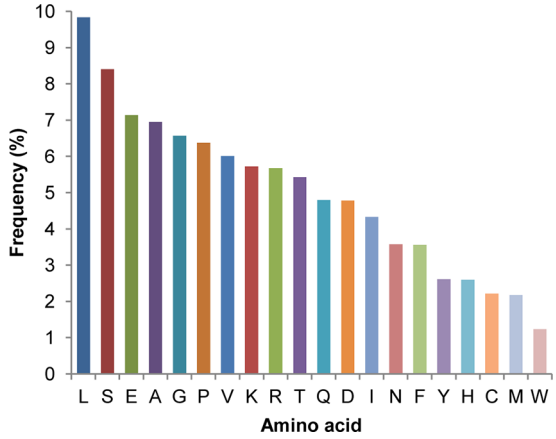

Figure 2. Amino acid composition of the human proteome. Amino acid abundances were obtained from the proteome analysis database.[47]

and are usually well distributed throughout a protein.[18] This leads to tryptic peptides with an average length of ~14 amino acids that carry at least two positive charges, which is ideally suited for CID-MS analysis.[20] The advantageous properties of tryptic peptides lead to high quality MS/MS fragmentation spectra and confident peptide identification in protein database searches. This in turn increases the accuracy of inference of protein identity.

Standardized protocols have been described for in-solution and in-gel protein digestion by trypsin (and other proteases).[21] A typical protocol involves denaturation of the protein using chaotropic agents like urea or guanidine, reduction of disulfide bridges using dithiothreitol (DTT), and subsequent alkylation of the cysteines by iodoacetic acid or iodoacetamide. After reagent removal and buffer exchange, the trypsin digestion is typically performed at neutral pH in an ammonium bicarbonate buffer at 37 °C. Depending on the way the digestion is performed, it may take up to 18 h (overnight digestion). The experimental conditions for trypsin digestion can be optimized for a specific application, for instance using a design of experiments approach.[22] The digestion is stopped by the addition of (formic) acid. Such digestion protocols are often very laborious and require many sample handling steps. High-throughput 96-well formats allow for automation of sample treatment steps via robotics.[23] Sample preparation for MALDI-MS analysis can also be easily automated by depositing (separated) proteins directly onto the MALDI target plate for on-plate digestion.[24] In-solution digestion may also be integrated into an online digestion LC system to increase throughput and reduce sample handling.[25] In addition, digestion may be achieved via immobilized enzyme reactors (IMERs)[26] or other formats, which are discussed below.

Despite the many advantages of trypsin, it may be necessary to use other proteases in specific cases, such as a lack or an overabundance of Lys and Arg in the protein sequence or pH incompatibility. A wide range of alternative proteases are available with different cleavage specificities, see Table 1. For instance, aspartic proteases like pepsin are active under acidic conditions and are thus often utilized in hydrogen/deuterium exchange (HDX) experiments that favor a low pH.[27,28] Pepsin proteases are less specific, but they allow reproducible digestion when five-to-six replicate digestions are performed.[28] However, a compromise is made due to the wider specificity or even nonspecific nature of the protease. Less specific proteases may lead to a larger number of peptides, even with overlapping amino acid sequences, which may result in more complex MS data. In addition, many smaller peptides may be formed, which are more difficult to annotate and are thus of little use for protein identification.

In contrast, the endoproteinases Arg-C, Asp-N, Glu-C and Lys-C provide high cleavage efficiency and specificity, and are often used as an alternative to trypsin. Like trypsin, Arg-C and Lys-C have the advantage to retain at least two basic amines in the peptide, N-terminal and Lys or Arg side chain, leading to doubly protonated peptides. Lys-N is the most recent addition to this group.[35] It has lower specificity and more observed missed cleavages than Lys-C, but it can be used under severe denaturing conditions, such as, elevated temperatures, in the presence of 8 M urea or 80% acetonitrile.[36]

Since the endoproteinases have a high selectivity for a single residue, they are often employed in middle-down proteomics. Global proteome and phosphoproteome analysis of whole cell lysates using Lys-N, strong cation exchange enrichment and ETD with supplemental collisional activation (ETcaD) may serve as an example of this.[37] As an alternative, limited proteolysis using common enzymes, for example, rapid digestion with trypsin, chymotrypsin or pepsin for only a few minutes, can be used to produce large protein fragments.[38] The recently introduced outer membrane protease T (OmpT), which cleaves specifically between two consecutive basic amino acid residues (Lys/Arg-Lys/Arg) and produces larger peptides (on average >6.3 kDa) than other enzymes, is also ideally suited for middle-down proteomics.[39] In addition, highly specific enzymes for a single (class of) target protein, such as the immunoglobulin-degrading enzyme of *Streptococcus pyogenes* (IdeS).[40] This bacterial cysteine protease specifically cleaves immunoglobulin G (IgG) under its hinge domain and cleaves the heavy chain into two fragments,[41] whereas the streptococcal cysteine proteinase streptococcal exotoxin B (SpeB from the

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Table 1. Commonly Used Proteases and Chemicals for Protein Digestion[a]

protease	organism	specificity	pH range	chemical	specificity	pH range
Arg-C	Clostridium histolyticum	R′	7.2−8.0[b]	CNBr	M′	acidic
Asp-N	*Pseudomonas fragi*	′D	7.0−8.0[b]	HAc	′D′[d]	acidic
Glu-C	*Staphylococcus aureus*	E′[b]	4.0−7.8[b]	FA	D′	acidic
Lys-C	Lysobacter enzymogenes	K′	8.5−8.8[b]	HCl	D′[e]	2.0[e]
Lys-N	Lysobacter enzymogenes	′K[c]	8.0[c]	NTCB	′C[e]	9 −10[f]
Trypsin	*Bos taurus*	K,R′	8.0[b]	Hydroxylamine	N−G	9.0[g]
Chymotrypsin	*Bos taurus*	F,W,Y′	7.0−9.0[b]			
Pepsin	Sus scrofa	′F,L,W,Y′	1.3			
		′F,L′	2.0			
Thermolysin	Bacillus thermoproteolyticus	′A,F,I,L,M,V	8.0[h]			
Papain	Carica papaya	R,K,D,H,G,Y[b]	6.0−7.0[b]			
Pronase	*Streptomyces griseus*	A,E,F,I,L,T,V,W,Y′	6.0−7.5[b]			

[a]All data obtained from the Expasy bioinformatics resource portal[29] (www.expasy.org), except those noted. [b]Roche Web site (www.roche-applied-science.com). [c]Raijmakers et al.,[30]. [d]Swatkoski et al.,[31]. [e]Smith,[32]. [f]Tang et al.,[33]. [g]Crimmins et al.[34]. [h]Sigma-Aldrich Web site (www.sigma-aldrich.com).

same bacterium) cleaves the heavy chains of all human immunoglobulins.[42] IdeS digestion of IgG results in three protein fragments of ∼25 kDa (the light chain, and the V_H−he_1 and CH_2−nd_3 domains of the heavy chain) that could easily be separated and characterized by LC-ESI-QTOF MS.

2.2. Nonenzymatic Digestion

Chemical cleavage is an alternative to enzymatic digestion. It can be achieved by treatment with dilute solutions of formic acid (FA),[43] hydrochloric acid (HCl),[44] or acetic acid (HAc),[31] or with other chemicals such as cyanogen bromide (CNBr),[45] 2-nitro-5-thiocyanobenzoate (NTCB),[33] and hydroxylamine.[46] These reagents have a high specificity for a single cleavage site (see Table 1) with similar abundances to Lys and Arg (in the case of Asp) or much lower (Met or Cys), see Figure 2.[47] Although the abundance is not necessarily correlated to the distribution of these residues in the sequence, chemical digestion may lead to high-mass peptides that are suitable for middle-down proteomics.[39]

Electrochemical oxidation is one of the latest additions to the array of nonenzymatic cleavage strategies, resulting in specific cleavage at Tyr and Trp.[48,49] Both these residues are low abundant in the human proteome, see Figure 2, at less than half of the occurrence of Lys and Arg in the entire Swiss-Prot database. *In silico* digestion of the proteins in the SwissProt database showed that the average size of electrochemically generated peptides would be 2.4 kDa,[48] thus ideally suited for analysis with a wide range of LC-MS instruments. Clear advantages of electrochemical cleavage of proteins are the speed of the reaction (minutes) and the possibility for online coupling to MS.[48] Electrochemistry, eventually online with MS, may also be used in the reduction of disulfide bridges, and thus may aid in achieving higher protein coverage.[50]

2.3. Multiple Digestion Strategies

Multiple enzyme digestion is a strategy to increase protein and proteome coverage through the utilization of a combination of proteases. The combined, parallel or successive use of multiple enzymes has been suggested as the only way to realize 100% sequence coverage.[51] Especially in whole proteome sequencing, the use of the multiple enzymes in parallel has significantly improved the number of identified peptides and proteins.[52] In this way, different portions of the proteome can be made visible, as demonstrated for complex samples like cerebrospinal fluid (CSF)[53] and plasma.[54] Several combinations of enzymes have been applied, although mostly trypsin is used in combination with an endoproteinase, that is, Lys-C,[54] Lys-N[55] or Glu-C,[53] or less specific enzymes.[56] Similar to multiple enzyme digestion, combinations of chemical and enzymatic treatments have also been reported and were found especially useful for the analysis of membrane proteins (see below).[57−59]

3. ACCELERATED DIGESTION TECHNIQUES

Bottom-up proteomics sample preparation protocols are often lengthy procedures, also requiring a number of pretreatment steps prior to protein digestion (see above). Although the sample preparation process can be automated, the digestion step remains the bottleneck in terms of time consumption. The advantages and drawbacks of various approaches for acceleration of bottom-up proteomics workflows have been reviewed several years ago.[60] In this chapter, approaches for acceleration of protein digestion in order to improve the throughput and recent applications of these techniques are briefly discussed. An overview of the accelerated techniques is given in Table 2.

3.1. Assisted Digestion

Whereas optimum digestion conditions, such as pH and temperature, have been established for many proteases and applications,[22,61] some proteases are known to perform well under a wider range of conditions. This has been catalogued in the BRENDA enzyme database (http://www.brenda-enzymes.org/). It is well-known that reaction rates improve at elevated temperature and, in some cases, a simple increase in digestion temperature may accelerate the protein digestion process. This may partially be caused by thermal denaturation of the protein, which increases the accessibility of cleavage sites.[62] Especially, the use of a thermostable protease allows for a significant increase in digestion temperature and a concurrent reduction in digestion time. For example, thermolysin digestion at 65 °C for 15 min or less resulted in unequivocal protein identification.[63] Reductive methylation of trypsin enhances the rigidity of its secondary structure and thereby increases its thermostability. This modification shifts the temperature for optimal catalytic activity to 50−60 °C, thereby allowing for faster digestion at elevated temperature.[64]

Obviously, not all proteases can withstand prolonged exposure to elevated temperatures.[65] As an alternative, microwave irradiation, which is known to efficiently accelerate organic reactions,[66] has already proven its usefulness in

Table 2. Overview of Techniques for Accelerated Digestion

accelerated technique	digestion time	online	compatibility	specific applications
High temperature	Minutes (~15)	Not done, but possible	Often applied to chemical digestion, not all proteases are thermostable	Wide application area
Microwave	Minutes (≤15)	Possible	Compatible with proteases and chemical cleavage reagents	Membrane proteins (increased solubility), glycoproteins (decreased sterical hindrance)
Ultrasound	Minutes (≤5)	Not feasible	Compatible with proteases and chemical cleavage reagents	Wide application area
High Pressure	Seconds (<60)	Yes	Mostly done with enzymes	HDX experiments (speed of online digestion-MS)
Infrared	Minutes (~5)	Not done	Only advantageous for enzymes due to increased interaction with protein	Wide application area
Solvent	Hours (≤5)	Not done, possible but requires stop-flow strategy due to long digestion time	Chemical digestion is often done in the presence of solvents, but some enzymes also tolerate relatively high percentages of organic solvent	Membrane proteins (increased solubility)
IMER	Minutes (≤20)	Yes	Compatible with each protease that retains activity when immobilized	Wide application area
Magnetic particle immobilized enzyme	Seconds (~30)	Yes	Compatible with each protease that retains activity when immobilized	Wide application area
On-chip immobilized enzyme	Seconds (5)	Yes	Compatible with each protease that retains activity when immobilized	Wide application area

accelerating protein proteolysis to several minutes, without significant protease degradation.[67] In comparison to a conventional incubation at 37 °C for 18 h, microwave-assisted tryptic digestion of a glycoprotein mixture was performed in only 15 min at 45 °C thereby achieving an even higher number of identified unique peptides and sequence coverage.[68] Alternatively, acid hydrolysis of proteins is generally performed at temperatures above 100 °C for several hours and is thus ideal to be microwave accelerated.[69] Recently, microwave-assisted acid digestion was combined with electrochemistry-MS to achieve a higher reproducibility and to introduce a third cleavage site, thus yielding smaller peptides with an average length of 10 amino acids, similar to tryptic digestion.[70]

Power ultrasound is known to have a positive effect on the reaction rate of various processes in the analytical laboratory.[71] High-intensity focused ultrasound can also assist in acceleration of digestion.[72] The digestion can be completed within minutes, even for complex samples, such as endothelial cell extracts,[72] soy bean protein extracts,[73] and bacterial protein extracts.[74] An evaluation of the effect of temperature on ultrasound-assisted digestion using an ultrasonic bath showed that ultrasound appears to be especially effective at temperatures below 55 °C.[75] However, it is know that ultrasonic baths are less efficient in the generation of sonic energy than sonoreactors or ultrasonic probes.[76] A comparison of the latter two methods showed that the sonoreactor has some advantages over the ultrasonic probe in terms of sample throughput and reproducibility, but both methods produce similar sequence coverages and numbers of peptide identifications.[76]

Although the exact mechanism of microwave-assisted and ultrasound-assisted digestion is still unknown, elevated pressure and temperature may play a role. Therefore, it may not be surprising that not only higher temperature,[64] but also high pressure has a beneficial effect on digestion efficiency.[65] Using a simple syringe to apply a pressure of 6 bar reduced the duration of a tryptic digestion to 30 min while obtaining similar or improved results as compared to atmospheric overnight digestion.[77] The beneficial effect of pressure may be due to increased protein denaturation since higher charge states were obtained for the intact MS analysis of myoglobin after application of 10000 psi (~690 bar) whereas a pressure of 35000 psi (~2400 bar) was demonstrated to reduce the tryptic digestion time of bovine serum albumin (BSA) to 60 s.[65] Pressure-assisted pepsin digestion of BSA or whole cell lysates at 25000 psi (~1725 bar) for 60 s improved the number of identified peptides and sequence coverage.[78,79] Online pressure-assisted digestion was also advantageous for HDX experiments in preventing back exchange of the reversible label.[80] In this study, proteins that are difficult to digest under normal conditions, such as amyloid β-peptide 1−42 and an HIV-1 capsid mutant protein, were successfully digested with pepsin in an online pressure-assisted digestion system employing a single UPLC gradient pump for digestion and delivering the sample to the MS for HDX studies.

Infrared energy has recently been suggested as a simple and reproducible means for accelerated digestion and was shown to improve protein identification for trypsin or chymotrypsin digestion compared to conventional digestion.[81,82] The beneficial effect of infrared energy on protein digestion may arise from increased interaction between enzyme and protein and higher exposure of the cleavage sites due to bond vibrations that fall within the wavenumber range of the infrared red.[82] A comparison between conventional and ultrasound- and infrared-assisted tryptic in-gel digestion showed that the accelerated techniques reduced the total sample preparation time from 19 to 3 h, while improving sequence coverage, number of identified peptides, and peptide scores.[83] In the study, infrared-assisted digestion was favored over ultrasound-assisted digestion because of shorter digestion time (5 vs 10 min) and slightly better Mascot results.

Solvent-assisted digestion has been described as well, but mainly in relation to membrane proteins (see below). High organic solvent concentrations, such as 80% of methanol or acetonitrile, decrease enzyme activity and may lead to irreproducible digestion,[84] but lower concentrations may have a beneficial effect on protein digestion due to improved unfolding of proteins while still retaining sufficient enzyme activity.[65] In the quantification of proteins in complex samples, solvent-assisted digestion was also shown to reduce the digestion time to 30 min and improve the throughput.[85] The digestion time may be further reduced by combining a solvent-assisted and a pressure-assisted digestion protocol, as was

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shown for the 60 s digestion of BSA in the presence of 20% methanol.[65]

3.2. Immobilized Enzyme Digestion

Improved throughput in protein digestion may also be achieved by immobilization of the protease onto a solid support.[86] Immobilization leads to increased enzyme stability through reduced autolysis and improved digestion efficiency due to the increased enzyme-to-protein ratio.[26] In addition, immobilized enzyme systems can be regenerated and reused for several times without loss of catalytic activity.[26]

In principle, any enzyme can be immobilized on beads made of different materials, including (activated) agarose,[87] polystyrenedivinylbenzene,[88] silica,[89] and glass.[90] The enzyme-immobilized beads may be used in an offline sample preparation method or packed into a column to be used in flow systems.[90] Most commonly, enzymes are directly immobilized in columns, also termed immobilized enzyme reactors (IMERs). This has been demonstrated for several enzymes, including trypsin,[91−93] pepsin,[94,95] chymotrypsin,[96,97] and alkaline phosphatase.[97] IMER digestion of a single protein can be achieved in less than 20 min with any of the aforementioned enzymes and is very versatile. IMERs can be used offline and even in tandem[97] but are also easily integrated into online systems,[98,99] for instance preceded by a protein separation step[100,101] or following the immunoaffinity extraction of a target protein from a complex mixture.[92,94]

For offline use, magnetic nanoparticles and polymer fibers for immobilized enzyme digestion have several advantages over the conventional solid supports, including a large surface area and a uniform and well-controlled size distribution, which improves enzyme loading and (apparent) activity.[102,103] After digestion, the magnetic beads are easily removed by means of a magnet. Their intrinsic properties, such as magnetism and conductivity, can be exploited in designing sample digestion protocols. Trypsin-coated magnetic nanoparticles in combination with elevated temperature have been employed in rapid digestion protocols requiring only 30 s for digestion of single proteins with high sequence coverage.[104] As magnetic particles are excellent microwave absorbers, microwave-assisted digestion using trypsin-coated magnetic particles allowed for rapid digestion of a fractionated rat liver extract within 15 s.[105,106] Pressure-assisted digestion involving trypsin-coated magnetic particles resulted in equal or better efficiency and reproducibility than conventional in-solution digestion.[107]

For online protein digestion in a capillary electrophoresis (CE) system, trypsin-coated magnetic beads were captured by two magnets.[108] Separated proteins to be digested were kept at the distal tip of a first CE capillary. Periodically, the resulting peptides are separated in a second CE capillary prior to MS detection. This two-dimensional system required 30 min for the digestion and separation of 30 protein fractions.

Polymer nanofibers are an alternative to beads. Trypsin-coated nanofibers were found to maintain a high activity level even after one year of repeated use and recycling.[109] Using nanofibers, a yeast proteome was digested in 6 h leading to the identification of 850 peptides corresponding to 400 proteins. As nanofibers have improved stability at high temperature and in the presence of solvents and various pH values, digestion under such rigorous conditions may reduce the required digestion time.[110]

Trypsin coated magnetic particles[111] and fibers[112] may also be used for on-chip digestion followed by MALDI-MS analysis. Alternatively, the microchannel of a microchip modified with zeolite nanoparticles can be used to immobilize trypsin.[113,114] This again provides accelerated digestion, for example, within 5 s for several standard proteins.[113] The microchip, online coupled to FTICR-MS, was applied to digest and separate a protein extract from a mouse macrophage. This led to the identification of 191 distinct proteins.[114] Even more complex protein analysis strategies may be performed on microfluidic devices, including integrated tryptic digestion, SPE enrichment, CE separation, and ESI−MS.[115]

4. DIGESTION STRATEGIES FOR SPECIFIC APPLICATIONS

Certain classes of proteins possess characteristics that prevent their analysis through conventional digestion approaches and require extra attention. Membrane proteins are notoriously difficult to solubilize and digest, which complicates their analysis with bottom-up proteomics.[116] On the other hand, the presence of post-translational modifications (PTMs), such as phosphorylation and glycosylation, may adversely influence the efficiency of the protein digestion as well as the ionization and fragmentation efficiency of post-translationally modified peptides.[117] Specifically designed protein digestion strategies for these protein classes are described below.

4.1. Membrane Proteins

Membrane proteins play an important role in cell signaling, cell−cell interactions, and transport across the membrane. Due to their hydrophobicity and limited solubility in aqueous solutions, they pose significant challenges to protein identification strategies.[116,118] Top-down proteomics seems to be ideally suited for the analysis of membrane proteins and their PTMs because the aqueous/organic solvent mixtures containing high acid concentrations (up to 90%) required for dissolution are highly compatible with ESI-MS.[119−121] However, as stated earlier, the size of the proteins may prevent full sequence coverage which may hinder complete mapping of PTM sites.

Bottom-up proteomics of membrane proteins is also not straightforward. As membrane proteins contain fewer Lys and Arg residues, tryptic digestion cannot effectively be employed in their bottom-up analysis. Improved sequence coverage of the membrane proteome can be achieved using other proteases like chymotrypsin and staphylococcal peptidase I[122] or pepsin.[123] A combination of chemical and enzymatic treatments was also found to achieve better sensitivity and membrane proteome coverage. Acidic conditions during in-solution chemical digestion improve the solubilization of membrane proteins and the resulting, more soluble, protein fragments can subsequently be digested with trypsin[57,58] or chymotrypsin.[57] In an in-gel digestion protocol for membrane proteins, trypsin digestion was followed by CNBr cleavage for improved coverage of the membrane proteome.[59]

Assisted digestion techniques (see above) can obviously be very beneficial in the digestion of membrane proteins. Microwave irradiation has been found to increase their solubility[124] and accelerate their digestion.[125,126] The solubilization of membrane proteins can be improved using detergents, such as SDS. However, detergents compromise the protease efficiency[127] and need to be removed prior to digestion and MS analysis, for instance using the filter-aided sample preparation (FASP) protocol.[128] Membrane proteins would more readily solubilize in organic-aqueous buffers and, as stated above, low

concentrations of organic solvent do not affect enzyme activity and may even have a beneficial effect on digestion efficiency. A methanol-assisted solubilization and digestion protocol was applied to the analysis of the membrane fraction of *E. coli* cells.[127] The solvent-assisted protocol, in which the membrane proteins were dissolved and digested in an ammonium bicarbonate buffer containing 60% methanol, was compared to a protocol using 1% SDS for solubilization and 0.1% SDS during digestion. The methanol-assisted method allowed for more protein identifications (358 vs 299), higher reproducibility, and more efficient identification of integral membrane proteins (159 vs 120). Moreover, the methanol-assisted digestion was completed in 5 h, compared to overnight digestion in SDS.

4.2. Post-translational Modifications

Microwave-assisted acid hydrolysis has demonstrated its usefulness for the simultaneous determination of the protein sequence as well as the identification of phosphorylation or glycosylation sites of several standard proteins.[129] This digestion technique was found to be especially beneficial for glycoproteins because the glycan moieties may sterically hinder enzymatic digestion.[68] Other assisted digestion techniques have not been applied to the study of protein PTMs. It is possible that the harsh conditions associated with these techniques, such as elevated temperature or pressure, may cause degradation and/or loss of the labile PTMs, although this did not seem to be the case for microwave-assisted digestion. Alternatively, specific enzymes (or chemicals) can be used to remove the PTMs prior to digestion of the protein backbone, for example, alkaline phosphatases and peptide *N*-glycosidases (such as PNGase F [Peptide-N4-(*N*-acetyl-*β*-glucosaminyl) asparagine amidase], PNGase A or endoglycosidases H or D).[130] A tandem microreactor setup, in which the first reactor contained immobilized chymotrypsin for protein digestion and the second reactor contained alkaline phosphatase for dephosphorylation, was successfully applied to the characterization of phosphoproteins.[97]

Advanced strategies for glycoprotein characterization have been described, including separate strategies to sequence the peptide backbone and to characterize the glycan structure.[131] For instance, CAD-MS/MS allows for sequencing of the monosaccharide residue, but (depending on the chosen sample pretreatment strategy) may not be able to pinpoint the site of the glycan attachment due to the loss of the sugar which is inherent to this type of fragmentation.[132] However, ETD fragmentation favors backbone cleavage of glycosylated peptides and, thus, does more readily allow for the identification of glycosylation sites.[132] As such, the application of middle-down proteomics, using the OmpT enzyme to generate larger peptides, was beneficial for the identification of post-translationally modified peptides.[39] Incomplete digestion with trypsin or Lys-C enhanced the determination of multiple phosphorylation sites of twenty recombinant nucleotide-binding proteins in *Escherichia coli*, including kinases and cystathionine beta-synthase (CBS) domain containing proteins.[133] Middle-down analysis of post-translationally modified proteins gives insight into the connectivity and combinatorial effect of multiple PTMs in the same polypeptide chain, which can be explored more fully with top-down proteomics.[7]

5. CONCLUSIONS AND PERSPECTIVES

Efficient and reproducible protein digestion is of utmost importance in bottom-up and middle-down proteomics. The selection of the most suitable enzyme or chemical reagent depends on the characteristics of the target proteins, such as amino acid composition and hydrophobicity, and compatibility with the digestion technique that is being used, in terms of pH or solvent compatibility. In addition, a wide variety of methods exist for acceleration of the digestion process to as less than seconds. Some of these techniques may be beneficial for certain applications, such as the digestion of membrane proteins. However, great care and consideration has to be taken to sufficiently optimize these accelerated digestion protocols, especially for protein or biomarker quantification, as they may lead to less reproducible results.

In-solution or in-gel protein digestion using trypsin in a bottom-up approach is still considered the gold standard in proteomics, although in particular applications, other enzymes or chemical digestion protocols may be useful. Middle-down analysis of proteins serves as a distinct alternative to bottom-up proteomics and has gained popularity in recent years due to the availability of specialized enzymes and continuous developments in MS instrumentation. The latter also enables the analysis of intact proteins in top-down proteomics, which is still somewhat limited in its application, but can provide information that is sometimes lost in digestion strategies. Selection of the suitable proteomics approach is guided by the types of research questions that need to be answered, the properties of the proteins to be analyzed, and the availability of expertise and instrumentation. In fact, due to their complementary nature, a combination of these approaches may be the only way to understand the biological processes that are essential to life.

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Notes

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